

Impaired growth and erythrocyte nuclear lesions of immature *Oreochromis niloticus* exposed to waterborne crude oil: Persistent responses

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Abstract

Crude oil impact studies have largely been limited to marine setting and it hampers the understanding and predictability pertaining to freshwater environments following contamination episodes. Growth and erythrocyte nuclear abnormalities (ENA) were followed in freshwater fish *Oreochromis niloticus* experimentally exposed to crude oil (Arabian light) dispersed in water. Study comprised of a control (no crude oil in water), and two experimental groups with 5 µL/L and 25 µL/L crude oil dispersed in water (5 ppm and 25 ppm respectively), and involved continuous exposure of fish (35 fish/tank in triplicate) with static renewal over a 90-day period. Growth was estimated serially at 18-day intervals (n=40-60). Both exposure groups reduced (p<0.05) weight-based and length-based growth rates and specific growth rates. Giemsa-stained peripheral blood and head kidney smears on day 90 (n=6) showed increased (p<0.05) micronuclei, nuclear buds, fragmented apoptotic nuclei and other types of ENA frequencies (per 1000 RBC) in the fish exposed to 25 ppm crude oil. Moreover, crude oil induced ENA levels were quantitatively different (p<0.05) between the peripheral blood and head kidney. Results show that crude oil hampers growth and sustains elevated ENA of *O. niloticus* juveniles in freshwater environment.

Keywords: Aquatic pollution; chronic toxicity; ENA; fish growth; head-kidney erythrocytes.

Introduction

Crude oil contamination adversely affects health of aquatic ecosystems. It is known that few millions of tonnes of crude oil enter maritime environment each year due to events such as natural seepages, tanker accidents and production water discharge at oil rigs. Contamination spreads to sediment and marine biota, and may drift into

inland waters as oil spills reach subtidal zones, intertidal areas and further into estuaries (Lee and Page 1997; Sammarco et al. 2013; Fry and Anderson 2014). Rivers and connected lakes remain vulnerable when crude oil mining or transportation takes place in river basins (Kochhann et al. 2015), while any other freshwater systems may be at risk because of accidental leakage or spills owing to human error.

Crude oil spills in aquatic environments often kill organisms *en masse* by entrapment in the slick and due to acute toxicity. Nevertheless, the damage on survivors and those that undergo contamination away from the slick may not be apparent. Although investigations that followed oil spills over years are seen in the field setting (Thomas et al. 1999; Irvine et al. 2006; Roberts et al. 2006; Sammarco et al. 2013), laboratory verification of long-term responses continues to be necessary for proper prediction of the aftermath. Controlled experiments remain important in this regard, as it allows results to be attributed to the exposure unambiguously excluding confounding effects of field heterogeneity.

The chemical composition of crude oil is complex and oil field-dependent while the impact assessment on constituent basis remains arduous. However, the whole crude as a toxicant mixture will depict a more realistic picture. Crude oil effects on teleosts span from cytochromes P450 gene induction (Carls et al. 2005; Brewton et al. 2013), oxidative stress (Roberts et al. 2006) and genotoxicity (Harvey et al. 1999; Pietrapiana et al. 2002) to growth retardation (Brewton et al. 2013), reproduction failure (Brannon et al. 2012) and teratogenicity (Heintz et al. 1999; Incardona et al. 2013). In addition, erythrocyte nuclear abnormalities (ENA) have been reported in finfish exposed to crude oil (Barsiene et al. 2006). Micronuclei and most ENA types contain cytoplasmic chromosome fragments or intact chromosomes that failed in proper segregation during mitotic cell division (Udroiu 2006). The increased ENA frequencies are symptomatic to genotoxicity and suggest exposure to genotoxic compounds (Çavaş and Ergene-Gözükara 2005).

Present study focuses on whether waterborne crude oil compromises growth, and genetic integrity measured as ENA in juvenile *Oreochromis niloticus*. The results will be valuable in assessing long-term crude oil impact scenario in freshwater environments.

Materials and Methods

Oreochromis niloticus fingerlings were obtained from the Fish Breeding Centre, Udawalawe, Sri Lanka. Following transportation, fish were acclimatized for two weeks in the laboratory experimental setup of identical fiberglass tanks containing 130L of aged municipal water with moderate aeration each. Triplicated setup included two treatments of crude oil (Arabian Light, Ceylon Petroleum Corporation) with 5 µL/L and 25 µL/L crude oil dispersed manually in water (5 ppm and 25 ppm (volume/volume) respectively), and a control (no crude oil). A number of 35 fish were assigned randomly into each of the nine tanks. Continuous exposure level and water quality were maintained by complete renewal of the experimental setup on each successive fourth day. Dissolved Oxygen, temperature,

pH and salinity were monitored on the third day from each renewal. Fish were fed throughout the study with formulated Prima Floating Foods® (Ceylon Aquatech Private Ltd, Sri Lanka) adjusted at 2% body weight (w/w) once a day.

Weight and length measurements were obtained from 20 random fish from each tank and serially with 18-day intervals over 90 days. On day 90, peripheral blood was drawn from the caudal vein of fish under anesthesia with benzocaine, and microscopic smears were prepared and stained in 10% Giemsa as described by Barsiene et al. (2006) before mounting in DPX medium. Pieces of head kidney (cephalic kidney) dissected out from fish were used to make a blood smear by pulling it gently on glass slide before staining and mounting in the same manner. Both peripheral blood and cephalic kidney mounts were observed under oil-immersion in a bright-field light microscope at 10x100 for enumeration of four categories of ENA, i.e. micronuclei, nuclear buds, fragmented nuclei and other altered nuclear morphologies (including blebbed, lobed, notched nuclei and binucleated RBC) as shown by da Silva Souza and Fontanetti (2006). Blind counts were taken up to a total of 5,000 erythrocytes per fish by the same observer.

Growth indices were estimated according to Hopkins (1992), and length growth rate (mm/day) and weight growth rate (g/day) were estimated for intervals between day one and respective sampling time points. Specific growth rates (SGR) of weight and length were calculated for 90-day experimental duration using $SGR_w = 100 (\ln W_2 - \ln W_1) / (t_2 - t_1)$ and $SGR_L = 100 (\ln L_2 - \ln L_1) / (t_2 - t_1)$ respectively where 2 and 1 represent final and initial status at times t_2 and t_1 .

Statistical analyses were performed using STATISTICA 7 software. Growth indices were tested by one way ANOVA among the groups within sampling time-points. ENA among experimental groups, and ENA between peripheral blood and cephalic kidney within groups were compared by non-parametric Kruskal Wallis and Mann-Whitney U tests respectively.

Results

Water quality was within the stated ranges across the tanks at monitoring time points throughout 90-day duration; temperature 28.2-28.3°C, pH 6.6-6.9, salinity 0.13-0.14 g/L and dissolved oxygen 3.36-3.43 mg/L. Both pre-exposure length and weight remained similar ($p > 0.05$) among all groups (Table 1) and subsequently showed an increasing tendency over the duration. However, as compared to control, waterborne crude oil affected ($p < 0.05$) total length as well as total weight since day 36. Fish showed reduced length- and weight-based growth rates ($p < 0.05$) in 5ppm and 25ppm groups (Table 2). Similarly, specific growth rates estimated for weight and length changes over 90 day period were lower in both exposed groups as compared to respective control (Table 3).

Table 1. Length (L, mm) and weight (W, g) of *Oreochromis niloticus* exposed to crude oil (mean \pm SD; n = 40-60)

day		Control	5ppm crude oil	25ppm crude oil	
1	W	7.68 \pm 2.66 ^a	8.43 \pm 2.82 ^a	8.16 \pm 2.78 ^a	
	L	75.95 \pm 8.93 ^a	77.82 \pm 9.93 ^a	77.82 \pm 8.63 ^a	
18	W	10.18 \pm 2.73 ^a	9.14 \pm 3.34 ^a	8.98 \pm 2.72 ^a	
	L	82.40 \pm 9.85 ^{ab}	83.66 \pm 10.11 ^a	78.89 \pm 9.99 ^b	*
36	W	11.32 \pm 3.49 ^a	9.67 \pm 3.74 ^b	9.42 \pm 2.93 ^b	*
	L	88.08 \pm 9.72 ^a	83.25 \pm 11.29 ^b	80.61 \pm 10.58 ^b	**
54	W	14.45 \pm 3.87 ^a	12.50 \pm 4.29 ^b	12.36 \pm 4.34 ^b	*
	L	97.18 \pm 8.95 ^a	92.23 \pm 10.40 ^b	90.69 \pm 11.06 ^b	*
75	W	16.34 \pm 3.92 ^a	13.52 \pm 3.67 ^b	12.81 \pm 4.77 ^b	*
	L	102.41 \pm 8.60 ^a	96.49 \pm 9.69 ^b	92.69 \pm 11.96 ^b	**
90	W	18.73 \pm 3.73 ^a	14.64 \pm 4.94 ^b	14.53 \pm 4.45 ^b	**
	L	107.46 \pm 6.55 ^a	99.97 \pm 11.66 ^b	101.79 \pm 9.67 ^b	**

*p<0.05, **p<0.001, one-way ANOVA followed by Tukey HSD where dissimilar superscripts indicate statistical significance among groups at a given sampling day

Table 2. Growth rates of *Oreochromis niloticus* exposed to waterborne crude oil.

day		Control	5ppm crude oil	25ppm crude oil	
18	WGR	0.138 \pm 0.033 ^a	0.039 \pm 0.025 ^b	0.045 \pm 0.006 ^b	*
	LGR	0.358 \pm 0.022 ^a	0.321 \pm 0.071 ^b	0.059 \pm 0.005 ^c	**
36	WGR	0.101 \pm 0.047 ^a	0.035 \pm 0.015 ^b	0.034 \pm 0.019 ^b	*
	LGR	0.336 \pm 0.082 ^a	0.149 \pm 0.014 ^{ab}	0.077 \pm 0.014 ^b	
54	WGR	0.129 \pm 0.023 ^a	0.075 \pm 0.009 ^{ab}	0.078 \pm 0.007 ^b	*
	LGR	0.394 \pm 0.068 ^a	0.264 \pm 0.043 ^{ab}	0.239 \pm 0.048 ^b	
75	WGR	0.118 \pm 0.019 ^a	0.068 \pm 0.017 ^b	0.061 \pm 0.016 ^b	*
	LGR	0.353 \pm 0.041 ^a	0.247 \pm 0.061 ^{ab}	0.197 \pm 0.057 ^b	
90	WGR	0.125 \pm 0.016 ^a	0.072 \pm 0.019 ^b	0.067 \pm 0.003 ^b	*
	LGR	0.350 \pm 0.027 ^a	0.244 \pm 0.048 ^b	0.245 \pm 0.006 ^b	

WGR= weight growth rate (g/day), LGR= length growth rate (mm/day), mean \pm SD (n=40-60) compared to day one;

*p<0.05, **p<0.001 one-way ANOVA followed by Tukey HSD where dissimilar superscripts indicate significant difference among groups at a given sampling day.

Table 3. Specific growth rates of *Oreochromis niloticus* exposed to crude oil.

	Control	5ppm crude oil	25ppm crude oil	
Weight based ^x	1.061± 0.165 ^a	0.673± 0.187 ^b	0.573± 0.165 ^b	*
Length based ^y	0.391± 0.039 ^a	0.278± 0.057 ^b	0.273± 0.008 ^b	*

^x SGRW = 100 (ln W₂-ln W₁)/(t₂-t₁); ^y SGRL=100 (ln L₂-ln L₁)/(t₂-t₁); given as % per day, over 90 days. *p<0.05 one-way ANOVA followed by Tukey HSD where dissimilar superscripts indicate significant difference among groups; mean ± SD; n=40-60

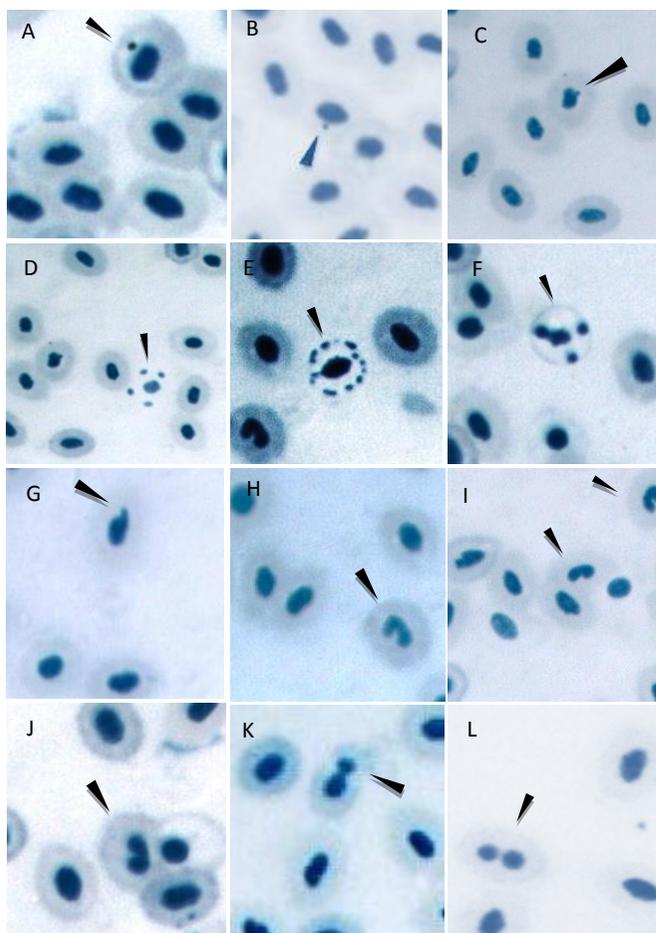


Figure 1. Erythrocyte nuclear abnormalities in peripheral blood of *Oreochromis niloticus* exposed to waterborne crude oil (arrows in A: micronuclei; B & C: nuclear buds; D, E & F: fragmented-apoptotic nuclei; and other altered nuclei including G: blebbed nuclei; H & I: notched nuclei; J & K: lobed nuclei, and L: binucleated RBC) (Images taken from Giemsa stained smears with 10x100 magnification and 5-10 fold appropriate digital enlargement).

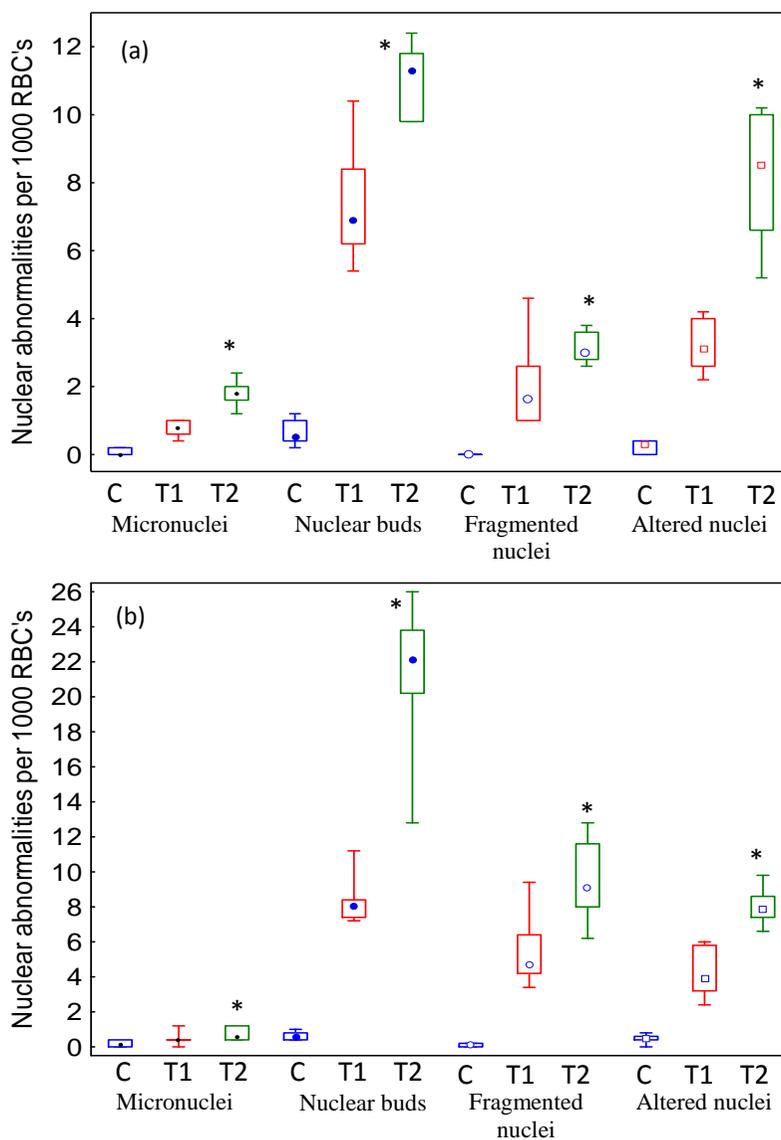


Figure 2. Erythrocyte nuclear abnormalities in (a) peripheral blood and (b) cephalic kidney of *Oreochromis niloticus* exposed to waterborne crude oil over 90 days (C: zero/control, T1 & T2: 5 and 25 ppm crude oil respectively; Data (n=6) presented as median, 25th and 75th percentiles (box), and minimum and maximum (whiskers); *Kruskal-Wallis test yielded $p < 0.05$ only between control and 25ppm group).

Long term continuous and renewed exposure to 25ppm crude oil dispersed in water increased the frequency of all forms of erythrocyte nuclear abnormalities ($p < 0.05$) counted in the study namely, micronuclei, nuclear buds, fragmented nuclei

and other types of abnormalities (hereafter referred to as altered nuclei, including blebbed, notched, lobed nuclei and binucleated cells; Figure 1) compared to control responses (Figure 2). The results show genotoxic impact of water borne crude oil on both peripheral blood and cephalic kidney erythrocytes. Fish exposed to 5 ppm crude oil did not however develop the abnormalities ($p>0.05$) at the same time point, day 90.

Intra-group comparison between peripheral blood and cephalic kidney with Mann-Whitney U test suggested that compartmentalized differences could occur in development of crude-oil induced ENA. Cephalic kidney showed higher ($p<0.05$) nuclear buds, and fragmented nuclei levels in 5ppm, and both 5ppm & 25ppm groups respectively. Control responses were not statistically different ($p>0.05$) between the tissues. Micronuclei showed increased level in peripheral blood as compared to cephalic kidney in 25ppm group ($p<0.05$).

Discussion

This paper addresses the areas of finfish growth and cytogenetic toxicity in a model freshwater fish species upon continuous exposure to crude oil in water over 90 days. The results showed growth retardation with certainty as total length and total weight growth declined as early as day 36 in such a way that it persisted throughout the experiment. Weight growth rate (g/day) and length growth rate (mm/day) estimated for contiguous periods, and specific growth rates (% per day) maintained by fish over entire experimental duration were affected as well. Comparable responses had been reported in other long-term experiments from both marine and freshwater finfishes previously. Those include weight reduction in juvenile pink salmon exposed 40 days to water soluble fraction (WSF) of crude oil (Moles and Rice 1983), reduced growth rate and survival of cutthroat trout exposed 90 days to crude oil (Woodward et al. 1981), and declined weight of Nile tilapia exposed to WSF over 10 weeks (Omoregie and Ufodike 2000). Decreased growth was also reported in polar cod fed with crude oil contaminated food (Christiansen and George 1995) and in larval Pacific herring exposed 16 days to weathered Alaskan crude oil (Carls et al. 1999). In feral pink salmon, embryonic stage exposure to weathered crude oil resulted in weight reduction following 200-300 days (Heintz et al. 2000). Brewton et al. (2013) reported growth reduction of spotted sea trout following 2010 BP/Deep Water Horizon oil spill in northern Gulf of Mexico. It is notable that the growth was affected in juvenile *Oreochromis niloticus* similar to marine fish responses to prolonged crude oil exposure. Although the exact mechanism remains obscure, the compromised growth could be a result of hindered molecular processes in tilapia development. The energetic costs in withstanding oil impact and in biotransformation of oil components cannot be ruled out as well.

Genotoxicity of crude oil exposure on *O. niloticus* juveniles evident in terms of erythrocyte nuclear abnormalities (ENA) both in peripheral blood and cephalic kidney are notable. The observation remains consistent with the previous reports on the increased ENA frequencies in teleosts exposed to crude oil (Barsiene et al. 2006), WSF of diesel fuel (Vanzella et al. 2007) and crude oil constituents such as PAHs (Al-Sabti and Metcalfe 1995). The fragmented nuclei in the study are

presumed to be apoptotic. Similarly, crude oil induced apoptotic red blood cells are known in experimental marine fish (Barsiene et al. 2006). It is plausible that ENA responses of tilapia in this study were persistent and sustained. The fish had been exposed continually over 90 days at the sampling point and it was unlikely that the ENA results were transient under such circumstances.

Statistically significant compartmentalized differences emerged in crude-oil induced ENA between peripheral blood and head kidney (HK) may show local influence on the responses. In tilapia, head kidney (pronephros) is a major site of hematopoiesis whereas the trunk kidney (mesonephros) functions primarily in excretion (Abdel-Aziz et al. 2010). The same authors maintain that head kidney accommodates various erythroblasts including young, mature as well as aging erythrocytes suggesting that it remains an active site of erythropoietic process. In this context, the differentiation and cell cycle activities such as DNA replication, chromatin condensation and nuclear formation as well as apoptosis may be commonplace in HK. In contrast, peripheral blood predominantly accommodates mature erythrocytes in circulation. Accordingly, toxicant induced compartmentalized ENA frequencies in HK may emerge different as compared to peripheral blood levels. Peripheral blood first encounters environmental toxicants across the gills and increased micronuclei frequencies evident in it is notable. Similar discrepancy of apoptosis induction in erythrocytes between HK and peripheral blood were reported in fish following three weeks of exposure to crude oil mixed with PAHs and alkylphenols (Barsiene et al. 2006), a toxic cocktail similar to produced water from oil platforms. ENA in teleosts are induced by unrelated agents such as laboratory genotoxicants (Ayllon and Garcia-Vazquez 2000, 2001), gamma radiation (Anbumani and Mohankumar 2012), heavy metals (Sanchez-Galan et al. 2001), polycyclic aromatic hydrocarbons (Oliveira et al. 2007), insecticides (Muranli and Güner 2011), petroleum refinery effluents (da Silva-Souza and Fontanetti 2006), and 17- β estradiol (Teles et al. 2006). The diversity among inducers may suggest multiple pathways that perhaps converge and trigger ENA induction.

In conclusion, continuous exposure to crude oil causes growth retardation and sustains ENA induction in *O. niloticus* juveniles in freshwater environment. Results promote the importance of contamination-free practices pertaining to petroleum-crude industries. This is of particular importance that *O. niloticus* is a major constituent species in the inland fishery of Sri Lanka, which forms a cheap source of animal protein for rural communities (Amarasinghe 2002, 2013).

Acknowledgements

All laboratory facilities and transportation/field costs provided by Department of Zoology, Faculty of Science, University of Ruhuna, Sri Lanka are appreciated. Comments from an anonymous referee were helpful in improving the text.

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